Variation in armour of three-spine stickleback

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Either I will find a way, or I will make one.

Acknowledgement

This thesis would not have been produced without my supervisors; Kjartan Østbye and Asbjørn Vøllestad.

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Abstract

The three-spine stickleback is an adaptable fish with variation in morphology and behaviour, inhabiting saltwater, brackish water and fresh water. It is armoured with 30-35 bone plates along its lateral line. In addition, it is equipped with three spines on its back and two pelvic spine. These features constitute an excellent anti-predator defence system. Yet, there is a strong selection for reduction in armour of three-spine stickleback in freshwater stickleback.

In this project, the bone structure and lateral plate of 72 three-spine stickleback from salt, brackish and fresh water was compared. Three-dimensional description of bone structures was accomplished by the use of micro-computed tomography (μ-CT). The results reveal that three-spine sticklebacks in freshwater may not only reduced number of plates, but also decreased the size and thickness of the armour plates.

Contents

Introduction

At approximately 10000 years ago, ice sheet that had been covering the landmasses in the northern hemisphere started to retreat. With the retreating ice, the ancestral marine three-spine stickleback (*Gasterosteus Aculeatus*) migrated into freshwater streams and colonized freshwater lakes. However, with the retreating ice releasing weight on the landmasses, the landmasses started to rise, leaving multiple populations of three-spine stickleback partially or fully isolated in freshwater lakes. The three-spine stickleback in saltwater, brackish water and freshwater show extensive variation in morphology, behaviour and diet (Wootton 1976).

In terms of morphology, the three-spine stickleback is a species with unique characters. Most notable is the heavily armoured features made of bone structures. The three-spine stickleback lacks fish scales and is instead armoured with up to 35 bone plates on each lateral side (Bell 1981; Reimchen 1983, 1992; Barrett *et al.* 2008). In addition, it is equipped with three dorsal spines and two pelvic spines. The dorsal and pelvic spines can be locked in an erect position, increasing the diameter of the fish. These features constitute an anti-predator defence. The dorsal and pelvic spines prevents a predator from swallowing it, while the lateral plates covering most of the fish body act as an armour against bite force (Hoogland *et al.* 1956).

The three-spine stickleback populations in freshwater and brackish water developed a parallel feature; a reduction in the number of bone plates covering its body (Bell 1994; Albert *et al.* 2008). This reduction in number of armoured plates was indeed not unique to one three-spine stickleback population, nor was it unique to one specific location (Albert *et al.* 2008; Schluter *et al.* 2010). This reduction in the number of bone plates repeatedly evolved parallel in freshwater three-spine stickleback populations all over the northern hemisphere, with a few exceptions (Cresko *et al.* 2004).

The reduction of lateral plates is occurring in a pattern that is categorized in distinct morphs (Bell 1976; Wootton 1976). There are three typical plate morphs with varying degree of plate loss; complete plate morph, partial plate morph and low plate morph (Fig 1.). The complete plate stickleback is the ancestral and original morph from the sea with full lateral plate coverage. The partial plated morph is equipped with plates at the anterior region and on the keel, but lacking one or more armour plates on the middle part or on the keel. The low plate morph have only anterior lateral plates, where the middle part and the keel are plateless. In addition, a fourth morph, which are completely plate reduced and with further reduction of armour by loss of pelvic girdle exist.

It has been shown that the ectodysplasin (*Eda*) gene is a major controller in development of number of lateral plates (Colosimo *et al.* 2004; Colosimo *et al.* 2005). A homozygote of the low plate (aa) *Eda* version will typically produce a low plate morph, while a heterozygote (Aa) results in a partial plate morph or a complete plate morph (Colosimo *et al.* 2004; Colosimo *et al.* 2005). Homozygote (AA) *Eda* is associated with the complete plate morph, however, several other linkage groups have been shown to affect both plate number and quantitative effects such as plate position and plate size (Peichel *et al.* 2001; Colosimo *et al.* 2004; Jones *et al.* 2012; Loehr *et al.* 2012). Populations of three-spine stickleback in freshwater may consist of a selection of plate morphs; a lake may include both low plate morph and partial morph, or only low plate morphs or only complete plate morphs or all three plate morphs may coexist in the same water.

Another important aspect of the development of the plates is plasticity. The lateral plates are not developed until the three-spine stickleback is 13 mm of length, being the last bone structures to be developed (Wootton 1976). Further the development of lateral plates, have been shown to be affected by rate of ossification, (Bell 1981), which is highly dependent on nutrient availability, in particular calcium .

Several hypothesis have been suggested to promote the armour reduction in freshwater and brackish water populations, such as; predator shift, swimming performance, buoyancy, flexibility, genetic linkage and mineral deficiency (Giles 1983; Reimchen 1983, 1992; Dalziel *et al.* 2012; Spence *et al.* 2012; Spence *et al.* 2013). Current research on armour reduction in the three-spine stickleback is predominantly focused on genetic mapping of plate reduction in plate morphs. While majority of freshwater three-spine stickleback populations are armour reduced with plate loss, there are some population that are not plate reduced. These populations with only the complete plate morph are assumed to lacking the *Eda* variant that allows for reduction of armour and thus are genetically locked to a complete armour.

However, if armour reduction is promoted by mineral deficiency, freshwater individuals that are locked to the complete late morph may not have sufficient minerals available and will not thrive. Unless there is another evolutionary path for armour reduction. Plasticity in bone structures in response to mineral deficiency is known in Zebrafish (*Danio rerio*), were mineral deficiency leads to small and porous bone structures (Siccardi *et al.* 2010). Similar, an alternative armour reduction may be decreased size and composition of the lateral plates.

To access such an alternative armour reduction, three-spine stickleback from populations of different salinity were compared by the use of micro-computed tomography (µ-CT).

The aim of the present study was to investigate variation in investment into bone structures in different populations of three-spine stickleback. I ask the following questions:

I. Do complete plated three-spine stickleback from populations in environments of different salinity (freshwater, brackish water and saltwater) invest differently into bone structures, such as total bone volume and size of lateral plate?

II. Do different plate morphs in a population differ in relative investment of bone structures, such as total bone volume and size of lateral plate? Is there any difference between plate morphs in freshwater and brackish water?

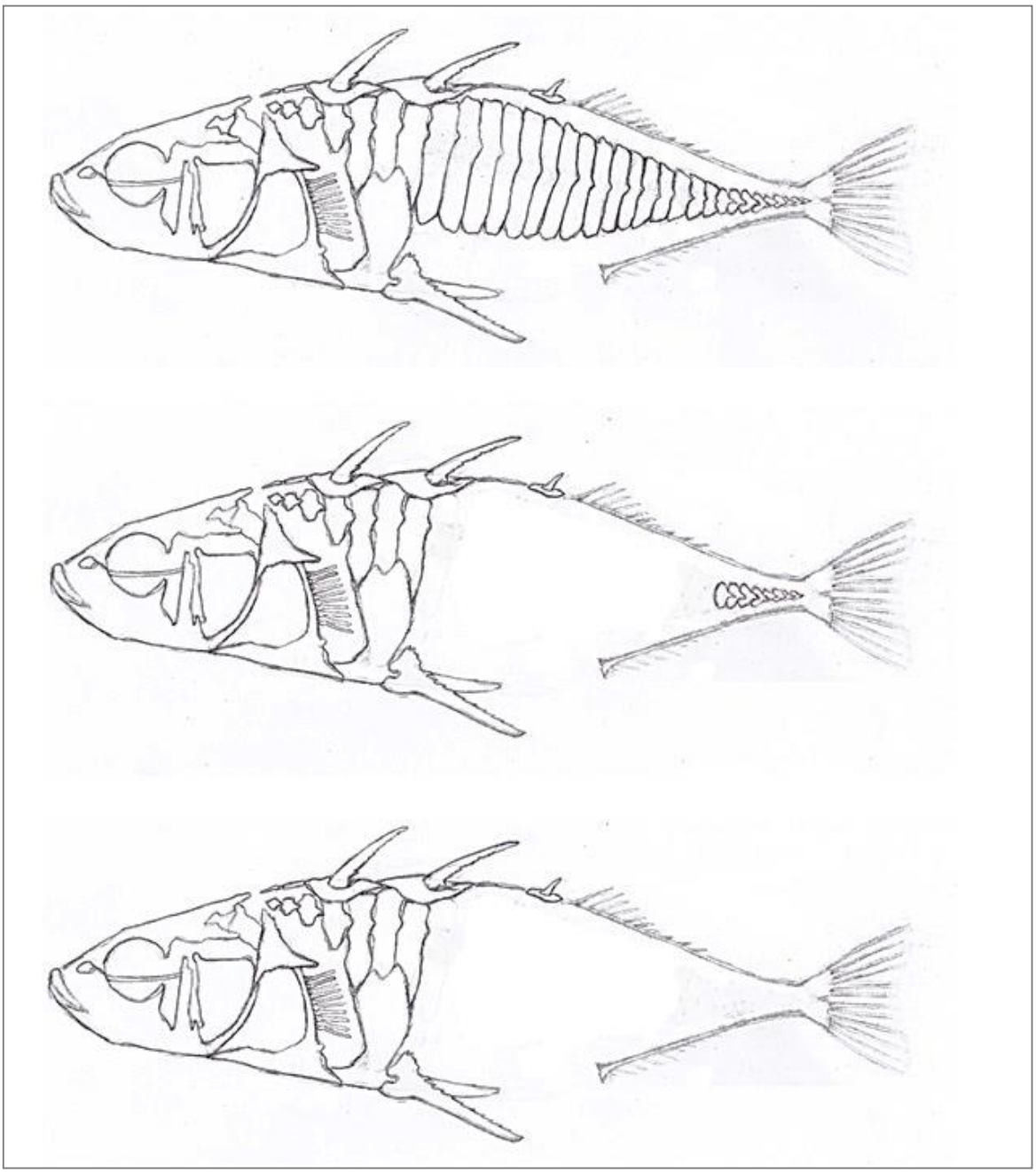


Figure 1: Lateral plate morphs in three-spine stickleback. Complete plate morph (top), partial plate morph (middle) and low plate morph (bottom).

Description of the bone amour of the three-spine stickleback

The armour of the three-spine stickleback consists of three different components (Fig 2). First, the three-spine stickleback has lateral plates that extend from the anterior part to the keel. These plates are connected with a joint, forming a solid armour, yet allows for some flexibility (Song *et al.* 2010).

The first anterior plates (LP1, LP2, LP3, LP4) are smaller than the other anterior plates, and less overlapping with the following lateral plate or not overlapping at all. Typically, a group three of the anterior plates (LP5, LP6, LP7) are underlying the first and second basal plate and the ascending process of the pelvic girdle. This overlapping construction makes a rigid belt supporting the rest of the armour (Reimchen 1983; Song *et al.* 2010). The group of lateral plates that connects with the basal plates and the pelvic are considered the most crucial part of the armour and these are the last plates to be reduced in armour reduction (ref).

The first plates in the middle part, typically LP8-LP14 cover the middle part of the fish. These plates are high with a rectangular shape. The following plates, typically LP15-LP24, cover from the dorsal fin to the keel. These plates are smaller with a more trapezoid shape. About eight to ten plates (LP25 to LP33-LP35), makes up the keel. These plates are not flat like the other dorsal plates, but have a bone protrusion at the middle of the plate. This bone protrusion overlaps with the following plates bone protrusion, forming a ridge.

Secondly, the three-spine stickleback is equipped with three dorsal spines. The spines are attached to basal plates with a connection and basal plate support the spine in an erect position (Reimchen 1983; Song *et al.* 2010). When not threatened, the dorsal spines are held down to the body.

In addition to the dorsal spines, two pelvic spines will also be locked in an erect position. The pelvic spines are attached to a pelvic girdle. The pelvic girdle has a pair of posterior process, which are fused together in a zipper like manner (Song *et al.* 2010). This posterior process covers the ventral part of the stickleback. When the spines are non-erect, they are lined up to the posterior process. On the left and right side of the pelvic, a pair of ascending process extends vertically, overlapping a group of lateral plates.

While the dorsal and pelvic spines are a defence against being swallowed, the lateral plates serve a function of shielding puncturing by bite force.

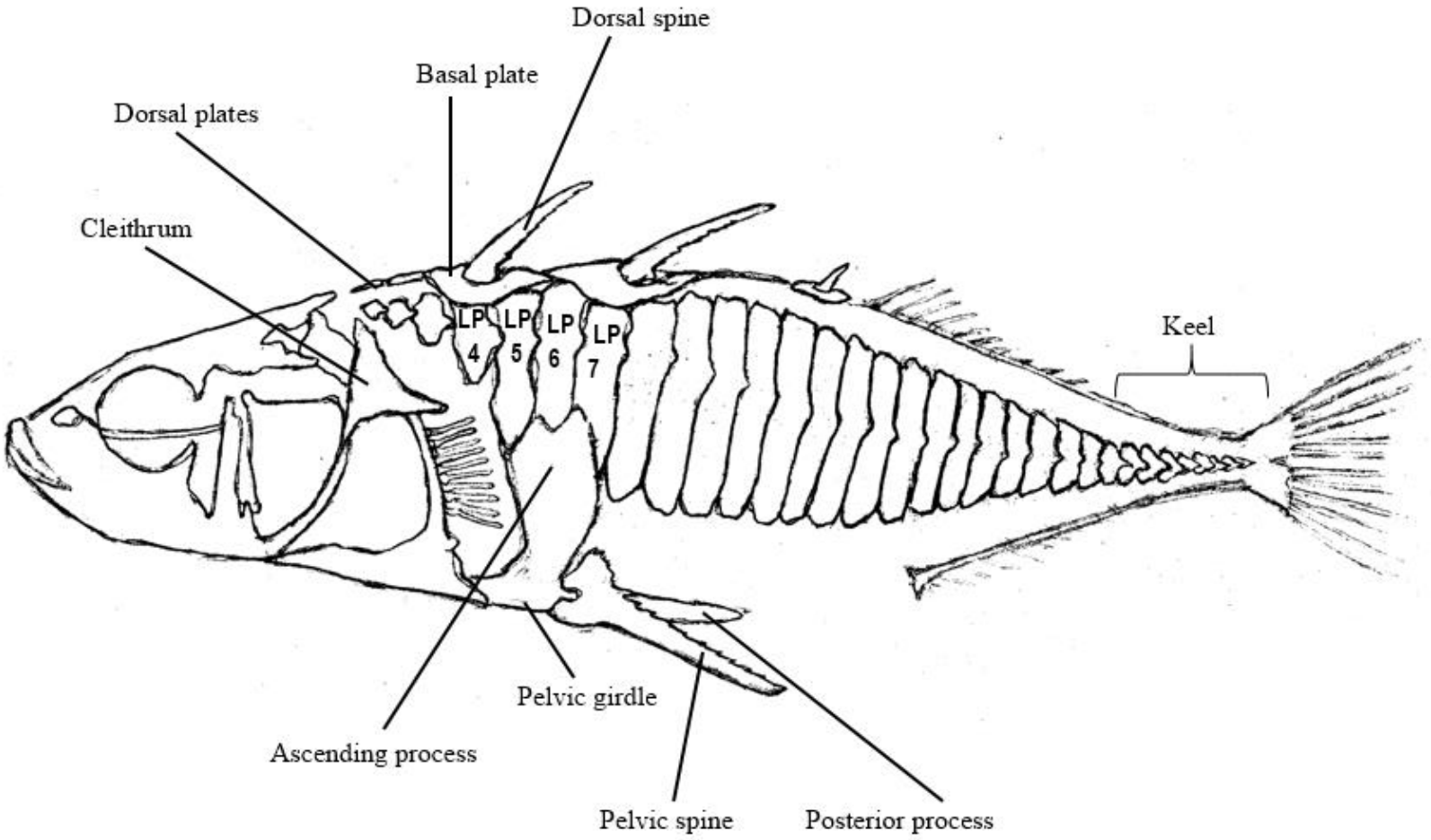


Figure 2: Armour components of a complete plate morph three-spine stickleback. Up to 35 lateral plates (LP) covers each side, the lateral plates at the tail region forms a keel. Three dorsal spines. The pelvic complex; a pair of pelvic spines attached to the pelvic girdle. The pelvic girdle has ascending and posterior process. Lateral plates (typically LP 5, LP 6 and LP 7) overlaps the basal plates of the dorsal spines and the ascending process of the pelvic girdle.

Materials and methods

A total of 72 three-spine stickleback were collected from four different locations, representing populations of different salinity habitats (Table 1). I sampled one salt water population (from the Barents Sea), one brackish water population (Engervann) and two freshwater populations (Asdøltjern and Gardatjørna). The three-spine stickleback from the Barents Sea and from Gardatjørna are fully plated; i.e. have no reduction in bone plate armour. These two populations represents the complete plate morphs and live at opposite ends of the salinity gradient. Three-spine stickleback in Engervann and Asdøltjern compromise variation in plate armour reduction, with all three plate morphs present; complete plate morph, partial plate morph and low plate morph. Including the morphs in the four populations, there is eight different groups with n=9 individuals in each group.

In the first part of the analysis, the complete plate morph from all four different populations were compared. In the second part of the analysis, the two populations containing all three plate morphs were compared.

Table 1: The mean length (cm) and standard deviation of three-spine stickleback from the four population samples. The complete plated morph was present in all populations, whereas the partially plated and the low plated morph was only found in Asdøltjern and Engervann.

	Complete plated	**Partially plated**	**Low plated**
Freshwater			
Gardatjørna	4.91 (0.52)		
Asdøltjern	4.93 (0.38)	5.27 (0.42)	5.14 (0.38)
Brackish water			
Engervann	5.23 (0.18)	5.16 (0.27)	5.03 (0.42)
Salt water			
Barents Sea	6.24 (0.36)		

Sample localities

Gardatjørna is a small and deep freshwater lake located in Fjell, Hordaland county (60°17’ 41.10" N, 05°05’09.81" E), 51 meters above sea level. This lake is isolated from other lakes as there are no connecting rivers. Sticklebacks were collected in July 2011 using hand-held dipnets and plexiglass traps (Breder 1960) baited with cheese set along the shoreline. Gardatjørna is possibly inhabited by trout (*Salmo trutta)*, which may predate on the sticklebacks.

Asdøltjern is a freshwater lake in Lier, Buskerud county (59°52’25.92” N, 10°19’47.10” E), 266 meters above sea level. Fish eating ducks and fish species perch (*Perca fluviatilis*), pike (*Esox lucius*), trout and whitefish (*Coregonus lavaretus*) may be predators on stickleback. Pike, roach and whitefish have been stocked in the lake recently (likely during the last 30 years) and are not the native fish fauna. Sticklebacks were collected in July and August 2011 using cheese-baited plexiglass traps and hand-held dipnets.

Engervann is a brackish water lagoon in Bærum (59°53′43.29″ N, 10°31′50.70″ E), Akershus county. The Øverlandselva river enters the lake, and a second river is connecting Engervann to the ocean through connection with the Sandvikselva River. During high tide, seawater highly influences lake Engervann, creating a fluctuating salinity level. There are also freshwater fluctuations in lake Engervann during the year due to rainfall and flooding in fall and spring. Eel (*Anguilla anguilla*), trout and salmon (*Salmo salar*) are commonly observed, with rare visitors of perch and european flounder (*Platichthys flesus*). Several fish-eating birds are also possible predators of the three-spine stickleback. Three-spine stickleback were collected during July 2011-May 2012 using cheese-baited plexiglass traps.

The Barents Sea sample was acquired from the Institute of Marine Research (Havforsknings-instituttet), in January-March 2012. Three-spine stickleback were sorted out from trawls at 255-387 meters depth (72°24′32.88″-75°26′54.00″ N, 30°18′52.96″-31°23′44.86″ E) frozen and stored on ethanol, before being transferred (shipped) to University of Oslo. The three-spine stickleback in the Barents Sea is expected to be under predation pressure of a wide variety fish that occupy the same part of the water column as the three-spine stickleback.

Sampling and handling of samples

Three-spine stickleback in Engervann, Asdøltjern and Gardatjørna were captured by plastic fry traps, gillnet or hand net. These collected fish were humanly killed by the use of an overdose of benzocain and stored in 97% ethanol.

In the lab total natural length was measured for each specimen (defined as length from tip of snout to end of caudal fin). Next, both left and right pectoral fins as well as the caudal fin were collected for molecular sex determination (and further DNA analysis).

Sex identification was done either by distinguishing male colour, visual examination of the gonads or by extracted DNA from fin clipping. DNA was extracted by standard DNA extraction method and amplified with primers for 3'UTR Idh (isocitrate dehydrogenase), a sex-determining gene (Peichel *et al.* 2004). Males were identified as heterogametic markers, with band at 302 bp and 271 bp, and females as homogametic marker with band at 302 bp, viewed on an ethidium bromide stained 5% agarose gel.

In order to the compare bone structure of three-spine stickleback sampled in different salinity environments, representative individuals were selected under specific criteria. These representative individuals were to be healthy and full-grown specimen. In populations containing three different plate morphs, ambiguous morphs, i.e., a specimen that would only lack one plate and thus be on the limit of a complete plate morph and a partial plate morph were excluded.

Minimum length was set to 40 mm. to ensure that all individuals were fully-grown and that bone plate development were completed (Wootton 1976; Bell 1981; Colosimo *et al.* 2005). Maximum length was set to 67 mm; this was due to the constraint of maximum size that could fit in the µCT scanner.

Injured fish were excluded, since different injuries like wounds may impact on bone structures. Further, heavily parasitized specimens were also excluded as several parasites that infect three-spine stickleback, like *Schistocephalus*, hinders natural foraging, swimming and behaviour. Thus, parasites may alter normal bone development.

Further, investment into reproduction, in particular female investment into eggs, may affect investment in developing bone armour structures. To exclude such investment, males were selected. However, the Barents Sea sample did not provide enough males, thus five non-gravid females were incorporated into the design.

Micro-computed tomography

Three-dimensional description of bone structures was accomplished by the use of micro-computed tomography (µ-CT). Samples were scanned with SkyScan 1172 micro CT, with a source voltage of 59 kV and with 167 µA source current and no filter. Samples were scanned with 15 µm voxel size.

The specimen was placed in an Eppendorf tube, to keep it stabilised during the scanning process. During test scans, fish were first fixed by modelling clay around the snout, which did not provide enough support during the scanning process and the fish would shift, due to drying, resulting in blurred scans. To place the specimen in an Eppendorf tube provided good scans.

The Eppendorf tube with the fish was mounted on a rotating platform in the scanner. When scanning the µ-CT takes series of cross-sectional X-ray pictures of the specimen along the longitudinal axis. These cross-sectional image slices are recorded every 0.5° while the specimen is rotating 360°. The results are a series of cross sectional image slices, approximately 3000-4000 slices, and these slices can be reconstructed into a 3D model of the whole specimen. Reconstruction was done by the use of SkyScan software NRecon. NRecon reconstruct a complete 3D model by stacking the image slices. When reconstructing, the model was corrected for beam-hardening (15%), which occurs when the X-ray beam is cone shaped and the photons passes differently in the middle and on the edge of the specimen (Bouxsein *et al.* 2010). A phantom sample, a model cylinder of a known density of about the size as the specimen, was scanned and reconstructed with the same values as the fish. The phantom is used for calibration when analysing tissue density (Bouxsein *et al.* 2010) and comparing structures.

A complete list of µ-CT scanning and reconstruction settings is given in the appendix.

To compare investment into lateral plates, one lateral plate (LP 4) was isolated from the reconstructed models. Bone structures can be separated with region of interest (ROI) using Skyscan CT Analyzer software. This was done by manually isolating the lateral plate from other bone structures on the image slice series. The isolated lateral plate (LP 4) was then saved as an added dataset for each fish. The same lateral plate, LP4, was chosen for all the individuals. This lateral plate was selected since it was developed in all plate morphs from all locations and did not extensively overlap with other bone structures. Overlapping lateral plates were more time consuming to isolate and human error was conciderered to be higher.

Reconstructed 3D models were viewed and visually described by using SkyScan CTvox software.

Structural parameters of the isolated lateral plate and whole specimen was abstracted from the data set using Batman, a batch processing software from SkyScan. The dataset was first cleaned and despecled. To exclude material that were not bone, such as the Eppendorf tube, lower grey thresholding value was set at 52, upper grey value was set at 255. This means that only tissue with a grey threshold higher than 52 will be used for analysis. The settings for the µ-CT analysis in CT Analyzer and Batman are given in appendix.

Several parameters can be abstracted from the dataset, both from the 3D model and in 2D and slice-by-slice model. Many of these parameters are not ideal for analysing the armour structures of the three-spine stickleback, due to the composition and structure of the bone. The number of parameters to be analysed was a trade-off between time and amount of data. The parameters chosen for statistical analysis were:

- Bone volume (mm^3): The total volume of bone. Bone volume was analysed for both the isolated plate and the whole specimen.
- Bone surface (mm^2): The surface area of the lateral plate (LP 4).
- Total porosity (percentage): The total percentage of open and closed pores in the lateral plate LP 4.

Statistics

Statistical analysis to compare the different populations and morph were done using anova and lm functions in R, with population and morph as factor, and with length or total bone volume as covariate. Model selection was done using anova model comparison function in R and simplest model that explained most variance was chosen.

All statistical analyses were done using R version 3.0.1 (R core team, 2013).

Results

Description of µCT model of three-spine stickleback

The µCT images of the three plate morphs of three-spine stickleback show that there is large variation in both shape and size of the plates both between the same plate on different individuals and between different plates on the same individual. Further, how neighbouring plates were connected (linked) also differed.

The lateral plates of complete plate morph individuals cover almost the entire lateral side of the fish (Fig 3). The lateral plates are formed as an outer armour; the plates seem to encase the sides of the fish (Fig 6). In comparison, the partial plate morph has reduced armour at the middle part, leaving an empty gap between the front lateral plates and the plates in the keel (Fig 4). This gap is particularly prominent in the dorsal view (Fig 6). The low plate morph only has lateral plates in the front (Fig 5), and the difference between the lateral plates encasing the complete plate morph and the low plate morph is very visible in the dorsal view (Fig 6).

Figure 3: A 3D model of a complete plate morph, three-spine stickleback. Lateral plates are covering from the anterior part to the keel. The lateral plates are of different shape in the front region, the middle region and the keel.

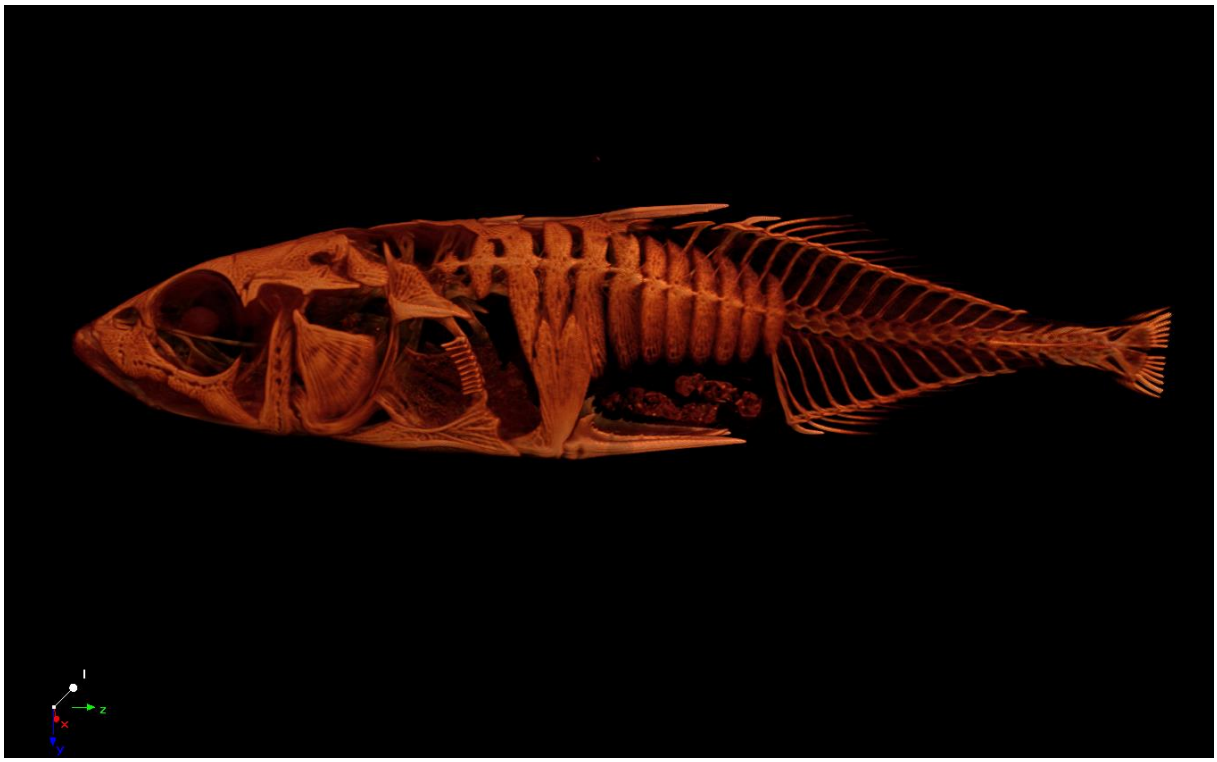


Figure 4: A 3D model of a partial plate morph, three-spine stickleback. Lateral plates are covering the anterior part and the keel, while the midsection is plate reduced.

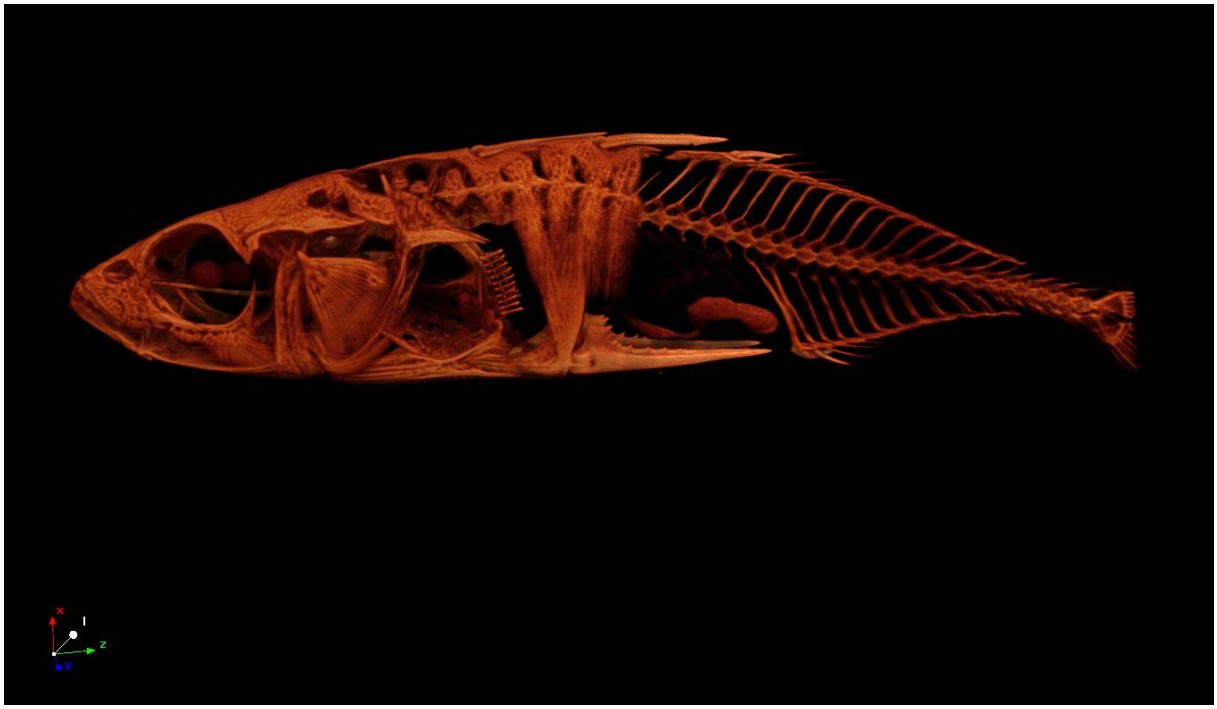


Figure 5: A 3D model of a low plate morph, three-spine stickleback. Lateral plates are covering the anterior part, leaving the middle and the keel bare.

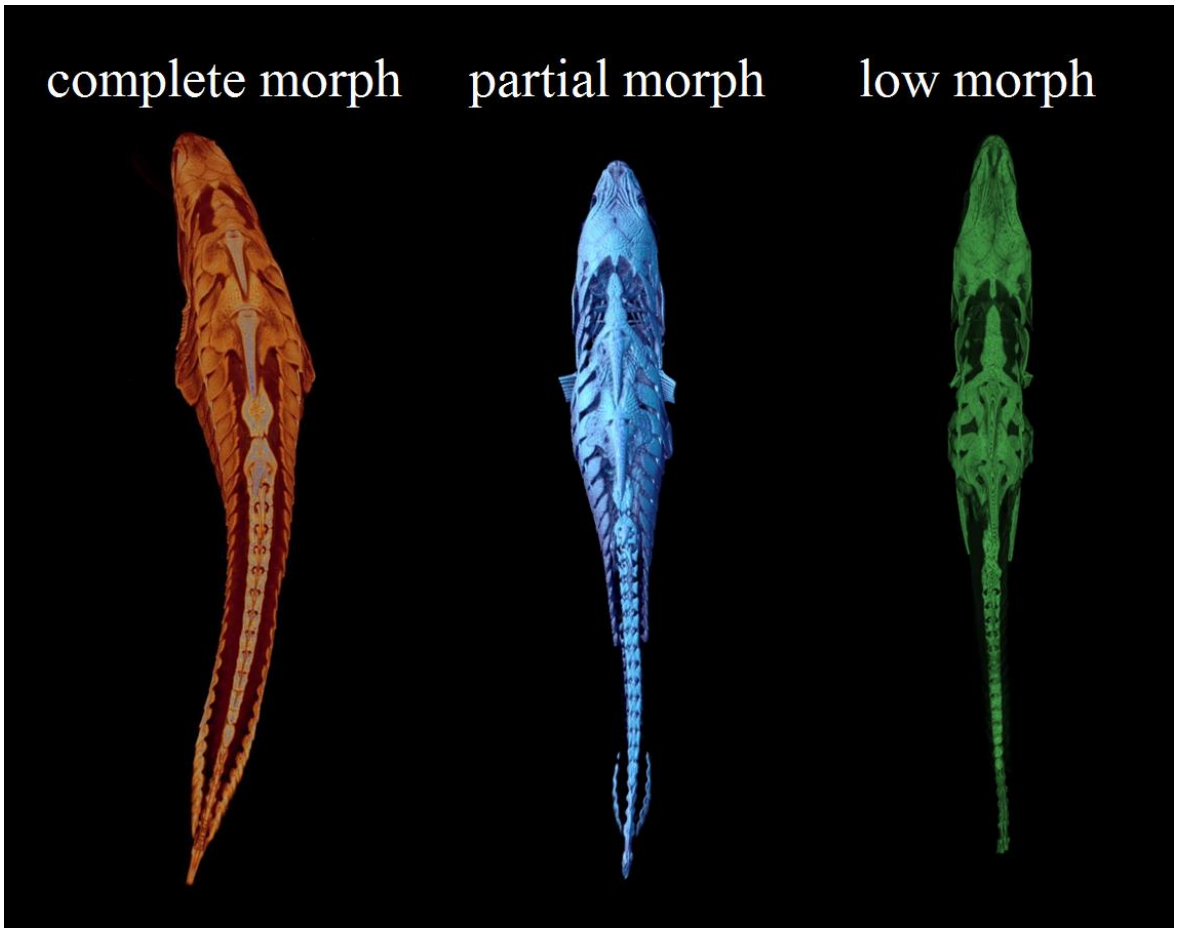


Figure 6: Doral view of the 3D models of a complete plate morph, a partial plate morph and a low plate morph of three-spine stickleback. The lateral plates encase the body of the fish.

Three-spine stickleback from the four populations varied in bone armour with differences in number of plates, size of plates, and shape and structure of plates. The complete plate morph individuals from the Barents Sea were heavily armoured with large lateral plates that covered a large area of the lateral side (Fig. 7). The first anterior plates (LP1-LP4) were positioned between the dorsal plates and the cleithrum. The isolated plate LP4 were underlying the basal plate. The lateral plates LP5-LP7, and sometimes LP4 and LP8, were overlapped by the ascending process of the pelvic and the basal plates. The lateral plates at the middle were uniform in size and shape and connected with joints.

In contrast, the plates of complete morph individuals from the freshwater populations were of different shape and size (Fig 8). The first lateral plates LP1, LP2 and LP3 were small and not bordering. Lateral plate LP4 were typically bordering, but not underlapping the first basal plate. Lateral plates LP5, LP6, LP7 and in some fish LP 8 were bordering or partially overlapped by the first and second basal plates and the ascending process. There were little

overlapping between these plates (LP5-LP8) leaving gaps between the lateral plates in the whole basal-, dorsal plate and pelvic connecting belt. The lateral plates covering the middle were smaller and less overlapping. In the extreme reduced individuals in freshwater, the lateral plates in the middle were reduced to thin strips, with large gaps between each plate.

Figure 7: The armour of a three-spine stickleback from the Barents Sea population. Lateral plates were large and overlapping

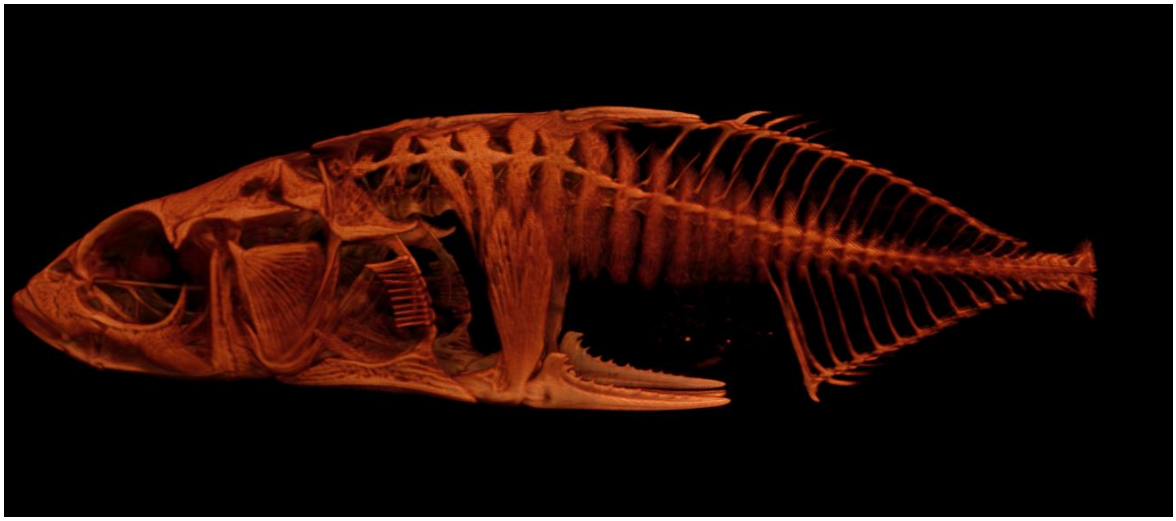


Figure 8: The armour of a complete plate three-spine stickleback from a freshwater population. Lateral plates were reduced in size, providing very little coverage.

The isolated lateral plate LP4 showed great variation in shape in all populations. Typically, three-spine stickleback from the Gardatjørna have rectangular shaped LP 4, this was also a common shape for the Barents Sea fish (Fig 7). In Engervann and Asdøltjern, the most common shape of LP 4 was a shark tooth shape as seen in Fig 3. An unusual, narrow shaped LP 4 was seen in complete plate morphs from Asdøltjern (Fig 8) and partial plate morphs from Asdøltjern and in a few low plate morphs from Engervann.

Comparison of the complete plate morph three-spine stickleback

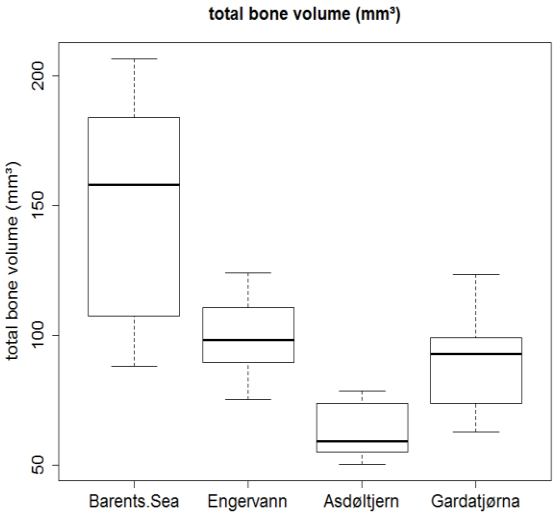
To compare relative investment in bone armour related to water salinity, complete plate morphs from the different populations were compared. The raw non-adjusted total amount of bone (mm^3) in the whole specimen was viewed for each population (Fig 9a). There was great variation between and within the populations. The Barents Sea fish had a mean bone volume of 149.5 mm^3, much greater than Engervann (99.6 mm^3) and the two freshwater populations (Gardatjørna 89.0 mm^3 and Asdøltjern 62.7 mm^3).

Similarly, an overall view of the isolated lateral plate in the four population showed that the Barents Sea sample differed from the other samples, both in terms of much larger volume (mm^3) and larger surface area (mm^2) of LP 4 (Fig 9b and 9c). The volume (mm^3) of plates ranged from 0.61-1.27 mm^3 in the Barents Sea, while Engervann, Asdøltjern and Gardatjørna ranged from 0.32-0.58 mm^3, 0.24-0.46 mm^3 and 0.31-0.89 mm^3 respectively

The fish from the different populations differed in size (Table 1), and as it is expected that bone mass increase with increasing size, length was used as a covariate when testing for variation in total bone volume (mm^3). Bone volume (mm^3) was significantly different in the population samples; the effect of length was also a significant (Table 2). In addition, there is a significant interaction between population and length. The total bone volume in the fish increased with increasing length, but at different rate in the different populations (Fig 10).

Bone volume and the surface area of in LP 4 was tested as a response to population and length (Table 3 and Table 4). The armour plate LP 4 increase in both volume (mm^3) and surface area (mm^2) with increasing length of the fish.

a)



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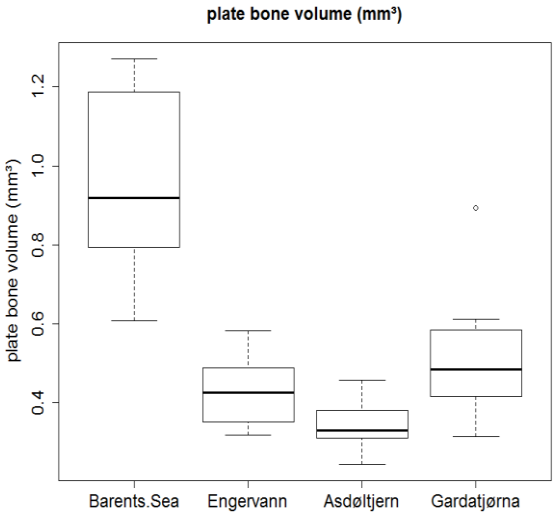

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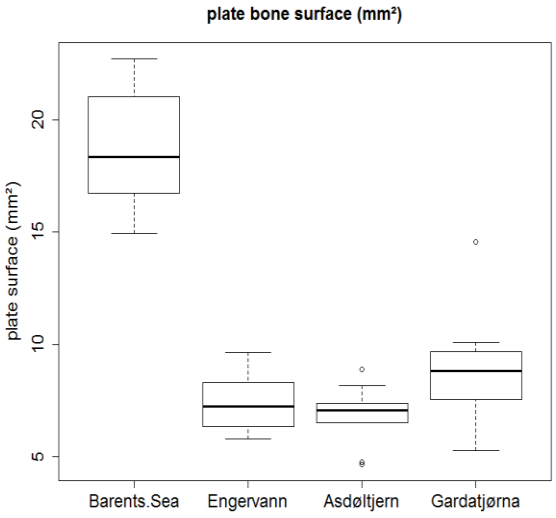

Figure 9: Box plot with the median, 25 and 75 percentile for a) the total bone volume (mm³), b) bone volume (mm³) in lateral plate LP 4, c) surface area (mm²) in lateral plate LP 4 in four populations of complete plate three-spine stickleback

The total porosity (percentage) of the plate was significantly different in populations, while there were no significant effect of fish length, on total porosity (percentage) of plate, there were however an interaction between population an fish length (Table 5).

In order to correct for length of fish, bone volume (mm^3) of plates was compared with the total bone volume (mm^3) of the fish (Fig 11). This model showed that there was a significant effect on plate bone volume (mm^3) by the total bone volume (mm^3) in the fish. There was a significant population effect, the interaction between total bone volume and population was not significant, but very close (P = 0.0867) (Table 6).

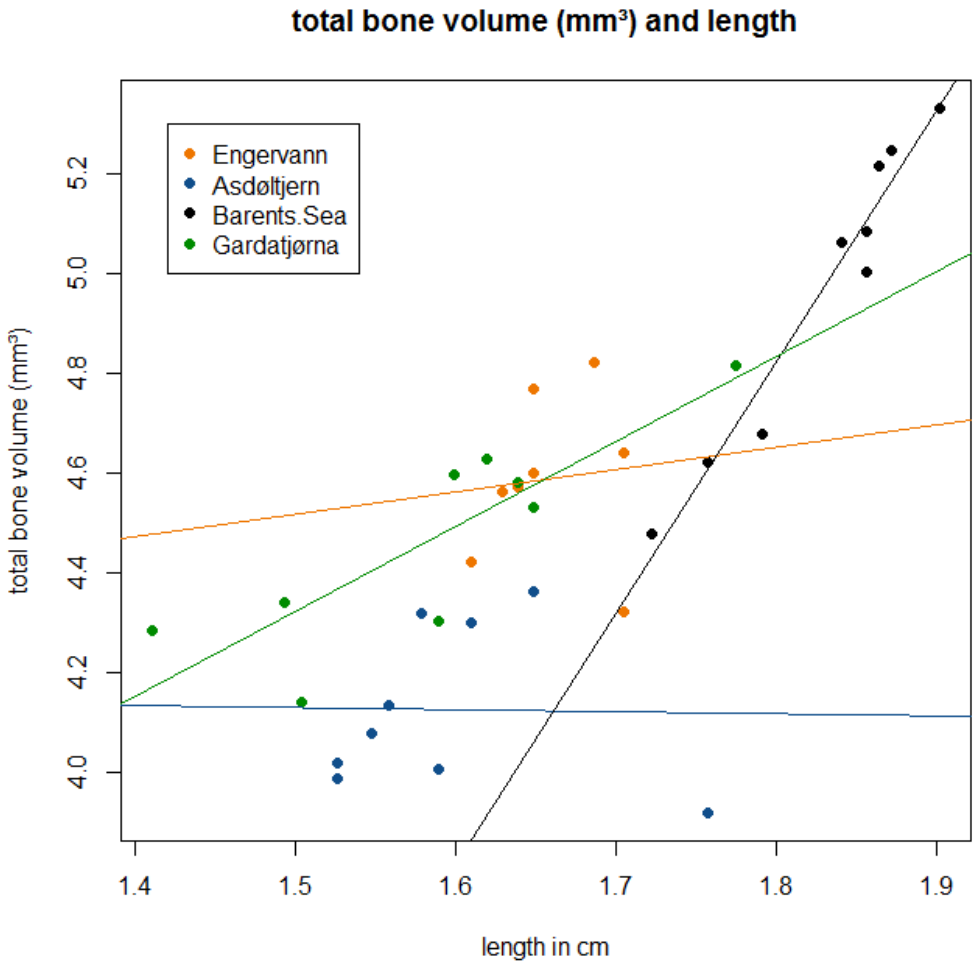

Figure 10: The relation between total bone volume (mm^3) and length in the population of four different populations of three-spine stickleback samples. Bone volume and length were log-transformed.

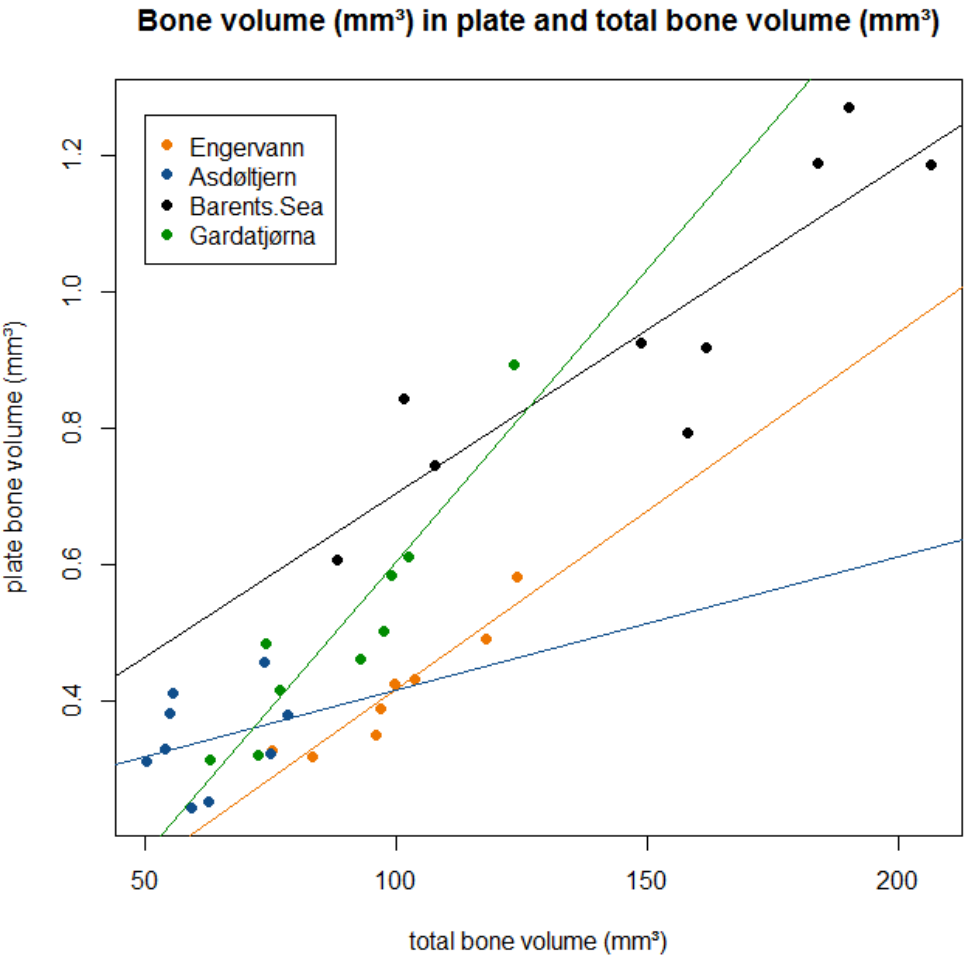

Figure 11: The relationship between plate volume (mm³) in LP 4 and total volume (mm³) in four populations of complete plated three-spine stickleback.

Table 2: Summary statistics for variation in bone volume (mm^3) in complete plate three-spine stickleback from four different populations: Barents Sea (salt water), Engervann (brackish water), Asdøltjern (freshwater) and Gardatjørna (freshwater) with length as a covariate. (N=35). Bone volume and length were log-transformed.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**P**
Population	3	3.263	1.088	54.54	<0.001*
Length	1	0.512	0.512	25.67	<0.001*
population:length	3	0.452	0.151	7.56	<0.001*
Residuals	27	0.538	0.020		

Table 3: Summary statistics for variation in bone volume (mm^3) of lateral plate LP 4 in complete plate three-spine stickleback from four different populations: Barents Sea (salt water), Engervann (brackish water), Asdøltjern (freshwater) and Gardatjørna (freshwater) with length as a covariate. (N=36). Bone volume and length were log-transformed.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**P**
Population	3	5.035	1.678	42.71	<0.001*
Length	1	0.795	0.795	20.24	<0.001*
Residuals	31	1.218	0.039		

Table 4: Summary statistics for variation in bone surface (mm^2) of lateral plate LP 4 in complete plate three-spine stickleback from four different populations: Barents Sea (salt water), Engervann (brackish water), Asdøltjern (freshwater) and Gardatjørna (freshwater) with length as a covariate. (N=36). Plate surface and length were log-transformed.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**P**
Population	3	5.974	1.992	81.31	<0.001*
Length	1	0.781	0.781	31.89	<0.001*
Residuals	31	0.759	0.025		

Table 5: Summary statistics for variation in porosity (percentage) of lateral plate LP 4 in complete plate three-spine stickleback from four different populations: Barents Sea (salt water), Engervann (brackish water), Asdøltjern (freshwater) and Gardatjørna (freshwater) with length as a covariate. (N=36).

	Df	**Sum Sq**	**Mean Sq**	**F value**	**P**
Population	3	489.2	163.08	3.906	0.019 *
Length	1	0.1	0.11	0.003	0.959
Population:length	3	440.5	146.82	3.516	0.028*
Residuals	28	1169.2	41.76		

Table 6: Summary statistics for variation in bone volume (mm^3) of lateral plate LP 4 in complete plate three-spine stickleback from four different populations: Barents Sea (salt water), Engervann (brackish water), Asdøltjern (freshwater) and Gardatjørna (freshwater) with total bone volume (mm^3) as a covariate. (N=36).

	Df	**Sum Sq**	**Mean Sq**	**F value**	**P**
Population	3	1.928	0.643	105.03	<0.001
Bone volume mm^3	1	0.547	0.547	89.37	<0.001
Pop:bone volume mm^3	3	0.045	0.015	2.43	0.087
Residuals	27	0.165	0.006		

Statistical comparison of the three lateral plate morphs within a brackish water and a freshwater environment

The two populations with three plate morphs were compared to investigate differences between morphs in freshwater and brackish water. First, the raw non-adjusted amount of bone (mm^3) in the whole specimen was viewed for each plate morph in both population (Fig 12). There is little variation between the populations, when not concidernig plate morph, only 10 mm^3 seperates the mean total bone volume mm^3, (68.4 mm^3) in Asdøltjern and (78.2 mm^3) in Engervann. Only one group, the complete plate morph from Engervann is standing out with a higher total bone volume (mm^3). One individual was (partial plate morph in Asdøltjern) is an outlier that much larger in terms of bone volume (mm^3). This individual is also of greater length, which from the first part of the results showed to correlate with bone volume, and therefore this outlier was not excluded.

Equivalent to the first part of the results, an overall view of the isolated lateral plate LP 4 in terms of bone volume (mm^3) and surface area (mm^2) was investigated in the two population and for each plate morph. From viewing the raw data, the bone volume (mm^3) of LP 4 did not seem to vary between the two population, and there seem only to be a morph effect, particulary in population Engervann (Fig 13.) There was no obvious trend viewing the raw data for surface area (mm^2) of LP 4 in Engervann and Asdøltjern (Fig 14.).

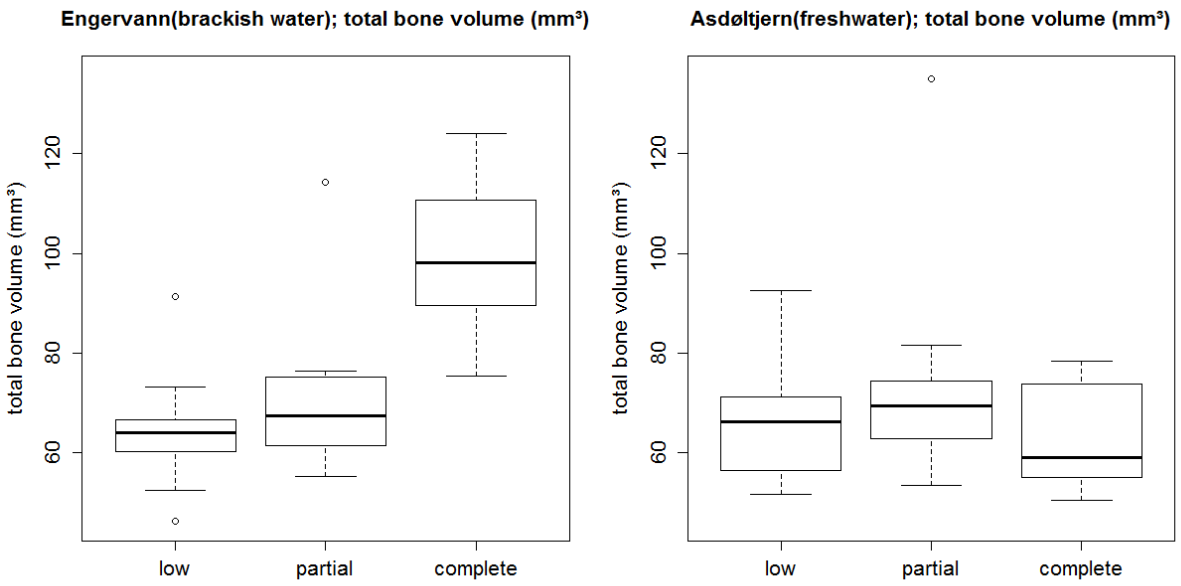

Figure 12:Box plot with the median, 25 and 75 percentile for the total bone volume (mm³) in three plate morphs in two populations; Engrvann and and Asdøltjern.

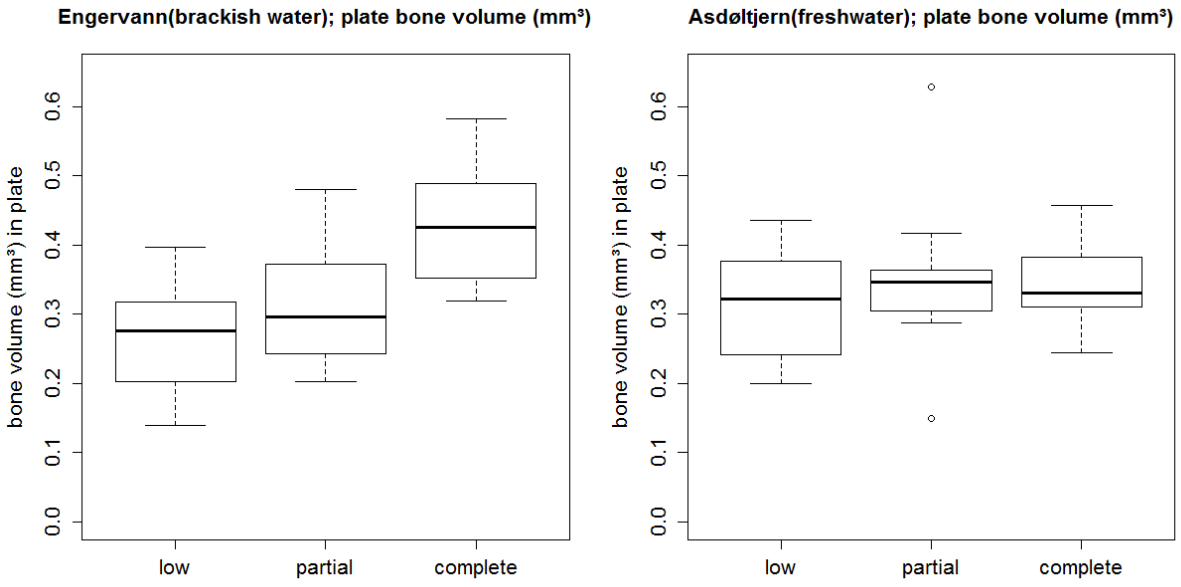

Figure 13: Box plot with the median, 25 and 75 percentile for the bone volume (mm³) in the lateral plate LP 4 of three plate morphs in population Engrvann and Asdøltjern.

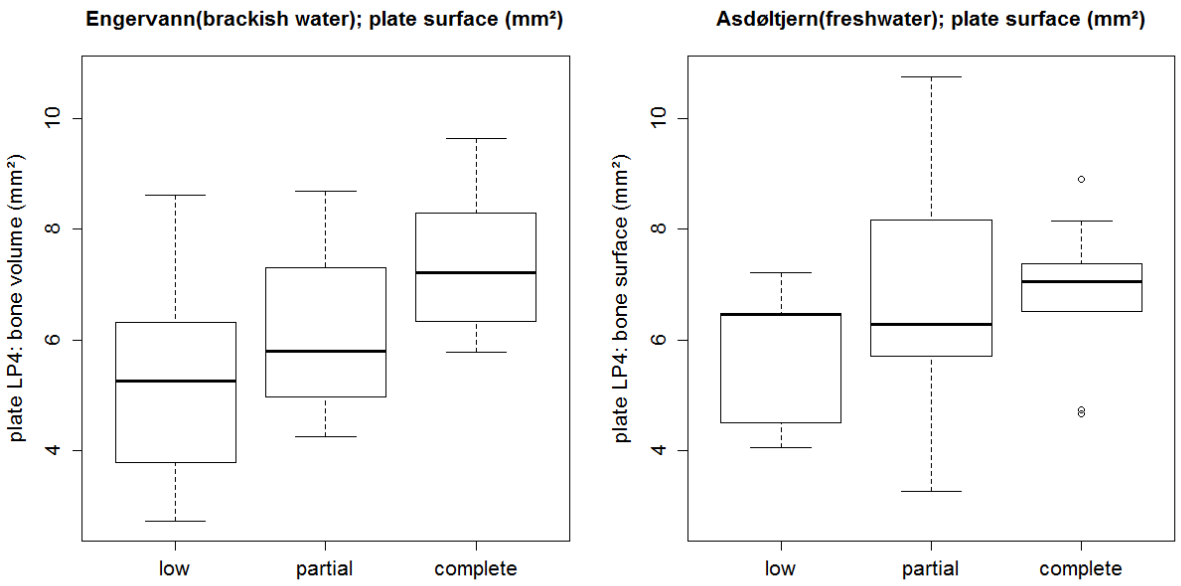

Figure 14: Box plot with the median, 25 and 75 percentile for the surface area (mm²) of the lateral plate LP 4 in three plate morphs in population Engrvann and Asdøltjern.

From the previous analysis it is known that the fish varied in length, and length was included as a covariate. The total bone volume (mm³) was significantly different in the two population, the different plate morph and length (Table 7). In addition there is an interaction effect between morph and population, and a morph and length interaction. Bone volume and length were log-transformed.

There was not a significant difference in bone volume (mm³) of lateral plate LP 4 between the two populations, but there is a significant morph and length effect (Table 8).

Similary, there is not a significant difference in surface area (mm²) of the lateral plate LP 4 in the two populations, however, there is a significant effect of both morph and length (Table 9). There is no significant interactions. Bone volume and length were log-transformed.

Just as in the first part, length was corrected for by plotting the bone volume (mm³) of the lateral plate against the total bone volume (mm³) (Fig 15). There is a significant effect of plate morph and total bone volume (mm³) (Table 10). However, there is not significant difference between the Engervann population and the Asdøltjern population.

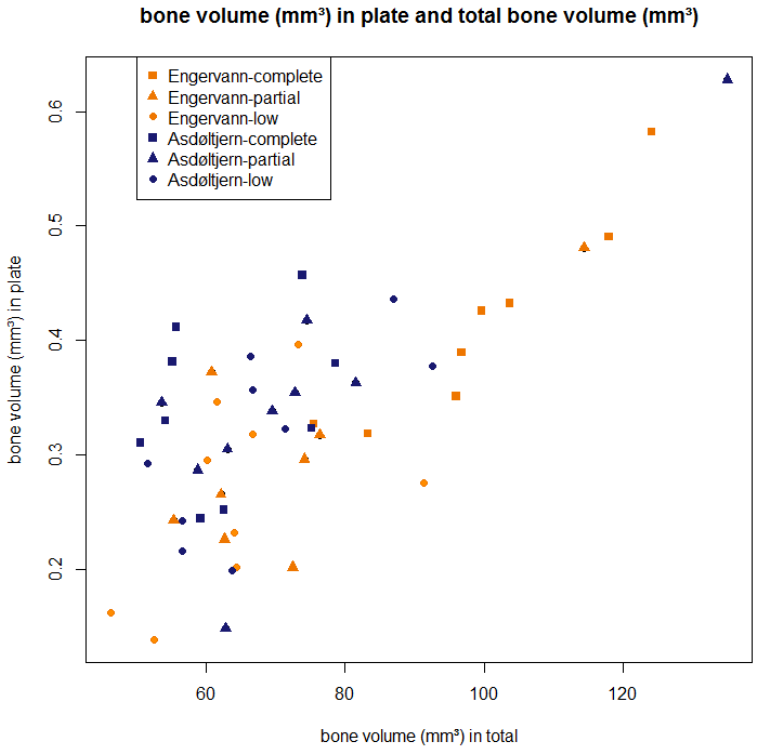

Figure 15: The relationship between plate volume (mm³) in LP 4 and total volume (mm³) in four populations of three-spine stickleback.

Table 7: Summary statistics for variation in total bone volume (mm^3) in different plate morphs of three-spine stickleback from two populations: Engervann (brackish water) and Asdøltjern (freshwater) with length as a covariate. (N=54). Bone volume and length were log-transformed.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**P**
population	1	0.196	0.196	7.665	0.008 **
morph	2	0.260	0.130	5.079	0.011*
length	1	1.040	1.040	40.693	<0.001**
population:morph	2	0.327	0.163	6.398	0.004 **
population:length	1	0.009	0.009	0.361	0.551
morph:length	2	0.255	0.127	4.983	0.012 *
pop:morph:length	2	0.001	0.001	0.012	0.988
Residuals	40	1.022	0.026		

Table 8: Summary statistics for variation in bone volume (mm^3) of a lateral plate LP 4 in plate morphs of three-spine stickleback from two populations: Engervann (brackish water) and Asdøltjern (freshwater) with length as a covariate. (N=54). Bone volume and length were log-transformed.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**P**
population	1	0.009	0.009	0.109	0.743
morph	2	0.846	0.423	5.375	0.008 **
length	1	0.759	0.759	9.644	0.003 **
Residuals	49	3.855	0.079		

Table 9: Summary statistics for variation in surface area (mm^2) of a lateral plate LP 4 in plate morphs of three-spine stickleback from two populations: Engervann (brackish water) and Asdøltjern (freshwater) with length as a covariate. (N=54). Surface area and length were log-transformed.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**P**
Population	1	0.008	0.008	0.147	0.703
Morph	2	0.769	0.384	6.898	0.002 *
Length	1	0.937	0.937	16.822	<0.001*
Residuals	49	2.730	0.056		

Table 10: Summary statistics for variation in bone volume (mm^3) of lateral plate LP 4 in different plate morphs of three-spine stickleback from two populations: Engervann (brackish water) and Asdøltjern (freshwater) with total bone volume (mm^3) as a covariate. (N=54).

	Df	**Sum Sq**	**Mean Sq**	**F value**	**P**
Population	1	0.002	0.002	0.621	0.435
Morph	2	0.068	0.034	8.685	<0.001*
Bone volume mm^3	1	0.255	0.255	64.889	<0.001*
Residuals	47	0.185	0.004		

Discussion

In general, the anti-predator armour of the three-spine stickleback varies considerably in different environment. The ancestral marine three-spine stickleback is heavily armoured, while the derived three-spine stickleback in freshwater is commonly reduced with regard to armour. Typically, this reduction of armour is seen as a decrease in the number of lateral plates; however, this does not exclude the possibility of alternative evolutionary pathways with regard to armour reduction. For example, changes in size and composition of these plates have yet to be explored. Here, one possibility is to reduce the amount of bone and minerals for bone building by having smaller lateral plates. In my comparison of a set of three-spine stickleback populations, the following questions were asked:

I. Do complete plated three-spine stickleback from populations in environments of different salinity (freshwater, brackish water and saltwater) invest differently into bone structures, such as total bone volume and size of lateral plate?

II. Do different plate morphs in a population differ in relative investment of bone structures, such as total bone volume and size of lateral plate? Is there any difference between plate morphs in freshwater and brackish water?

The results of the comparison of only the complete plated three-spine stickleback in salt water, brackish water and freshwater environment indicated great differences in the total bone volume (mm^3) of the fish. The Barents Sea individuals had significantly larger total bone volume (mm^3) than individuals from brackish water and freshwaters. In addition, lateral plate (LP 4) was significantly larger in terms of the surface area (mm^2) and the bone volume (mm^3) in the Barents Sea population than in the brackish water and freshwater populations.

A comparison of the two populations (freshwater and brackish water) both holding each of the three plate morphs showed very little difference in the overall mean of the total bone volume (mm^3). In addition, there were little differences overall in the size (both surface area mm^2 and bone volume mm^3) of the lateral plate LP 4. Thus, there seems to be little overall difference in size (surface area mm^2 and bone volume mm^3) of lateral plates in the different plate morphs in freshwater and brackish water.

These findings will be discussed in more detail below.

Variation in armour of the complete plated three-spine stickleback in different salinity regimes.

The comparison of only the complete plate morphs in different salinity regimes depict more than just the effect of salinity on development of lateral plates. The alternative armour reduction by decrease in size of plates may be a trait that is genetically linked to reduction in number of plates (Leinonen *et al.* 2012). In this comparison of populations, there is one population that is seemingly only complete plate morph: Gardatjørna (freshwater). If decrease in size of lateral plates is linked to reduction of plates, this population is expected to have lateral plates that are not decreased in size, based on the assumption that this population does not have the genetic potential for plate reduction.

Interestingly, the freshwater population with only the complete plate morph observed (Gardatjørna), had similar small sized lateral plates as seen in the freshwater and brackish water populations where sympatric plate reduced morphs (partial plate morph and low plate morph) existed. The individuals in Gardatjørna had similar small lateral plates (both surface area mm^2 and bone volume mm^3) as in Asdøltjern (freshwater) and Engervann (brackish water) populations. These results support the proposition of an alternate path to armour reduction, either by phenotypic plasticity or by natural selection and suggest that the alternative path of armour reduction by having smaller plates is not directly linked to armour reduction by reduction in the number of plates. This finding is supported by a study by Leinonen *et al.* (2012), were a freshwater population of the three-spine stickleback appeared to be homozygote for the complete plate morph *Eda* allele (AA). This population had exceptional small lateral plates, even smaller than in populations with the recessive low plate allele (aa) (Leinonen *et al.* 2012). Further, in a study by Myhre and Klepaker (2009), a mass measurement (grams) of individual lateral plates from complete plated freshwater stickleback and complete plated marine stickleback, showed that the marine stickleback had significantly greater mass of lateral plates than the freshwater specimen did.

The development of the small lateral plates in the complete plated freshwater individuals is possibly due to phenotypic plasticity or due to natural selection. These alternatives cannot be differentiated in my study, but should be approached by conducting a common-garden experiment using the same populations under variable water chemistry scenarios in order to test if water conditions affects the lateral plates.

Contrary to my expectations, results showed that the brackish water population Engervann was not intermediate in lateral plate size and total bone volume between the marine and the freshwater populations. Instead, the total bone volume (mm^3), and size (surface area mm^2 and bone volume mm^3) of lateral plate LP 4 were similar to the two freshwater populations. However, since there is only one brackish water population in this study, this conclusion is only speculative, and should be investigated further. It should be noted that although the salinity of Engervann is likely fluctuating due to tide and rain influence and the sampling occurred over several months, I assume that the average salinity is still low compared to other brackish waters.

Another implication for comparison of these populations is variation in length. The fish varied in length; in particular, the Barents Sea population were of greater length than the other populations. Although mass of lateral plates is related to body size (Myhre & Klepaker 2009) and the minimum length set assures fully developed lateral plates (Wootton 1976; Bell 1981) it cannot be excluded that further ossification of lateral plates occurs at a different rate in different populations (Bell 1981; Marchinko & Schluter 2007; Cresko 2008). Secondly, it is not known if the fish are in the same age class, as growth rate and average life span is different in marine and freshwater sticklebacks (Bell 1994; Guderley *et al.* 1994). Since plate mass development is associated with length of fish and both growth rate and plate development have been associated with mineral abundance and salinity (Spence *et al.* 2012), both length of fish as well as plate development is depending on mineral levels. Thus, the decrease in lateral plate size and decreased fish length is a natural coherence and when comparing lateral plates in different salinity fish should not necessary be of equal length.

<u>Variation in armour between different plate morphs of three-spine stickleback in a freshwater and brackish water population.</u>

This comparison are of a particular interest, both populations; Engervann and Asdøltjern have plate reduction occurring, as seen in different morphs, but have different salinity. If decrease in plate size is linked to plate reduction, a variation in plate size is expected to occur the different plate morphs. Although there was a significant plate morph effect, the plate morph effect were only seen in population Engervann. The complete plate morph in the brackish water population Engervann had larger sized lateral plate (LP 4) and greater total bone volume (mm^3) than the partial plate morph and the low plate morph. If decrease in size of

plates were linked to plate reduction, the expected results would be levels of lateral plate size decreasing with each plate-reduced morph in both populations.

A possible explanation for these results might be that a part of the population in Engervann are migratory marine individuals. Such, anadromous individuals could obtain more minerals and energy needed to build larger bone structures by feeding in the oceanic environment than sticklebacks being retained in the brackish water environment in Engervann. A study by (Bjærke *et al.* 2010) conducting a foraging experiment using live *Daphnia* spp. and dead bloodworms, showed that the complete plate morph was more efficient feeding on *Daphnia* and that the low plated morph was more efficient feeding on bloodworms. The partially plated morph were between the complete plate morph and the low plate morph with regard to preferences and feeding efficiency. Thus, the three lateral plate morph in Engervann had different foraging behaviour and likely prey preferences, which could reflect that the complete plated morph is anadromous. An unpublished study (Østbye *et al.*) using stable isotopic signatures on prey items in Engervann and in the ocean, as compared with stable isotopic signatures in the muscle of the three lateral plate morphs in Engervann showed that the complete plate morph were likely more anadromous foraging on pelagic crustaceans at a higher trophic level. In contrast, the low plated morph appeared more benthic with regard to foraging, suggesting it was more likely to be foraging in Engervann than being anadromous.

Further, it is interesting that there is not a significant difference between the two populations. As previously discussed, the results of Engervann population having similar total bone volume (mm^3) as well as size of lateral plate LP 4, is unexpected. If decrease in size of plates is a reaction to different salinity, it would be expected that the decrease in plate size occurred at different level, small size plates in Asdøltjern (freshwater) and intermediate size plates in Engervann (brackish water) respectively.

Analysis of findings in 3D models

Song *et al*. (2010) applied µCT scan to create 3D models of parts of one marine three-spine stickleback individual. The dorsal spines, basal plates, lateral plates and the pelvic girdle with spines were scanned and later analysed. Although, Song *et al*. (2010) had different lateral plates isolated for analysis (LP 9, LP 10 and LP 11) their reported measures of bone volume (mm^3) is within the same range as the Barents Sea in this study. Similar, the description of

bone structures, in particular lateral plates is comparable with descriptions of the typical Barents Sea individuals in this study.

From visual analysis of the individuals in this study, the lateral plates from anterior to the keel were different in size and shape. In the Barents Sea, the lateral plates in the middle were rectangular shape bordering neighbouring plates with a protruding bone bridge connecting them. This shape of lateral plates in marine three-spine stickleback is reported from scanning electron microscope (SEM) studies (Lees *et al.* 2012) and µCT studies (Song *et al.* 2010).

From the µCT images and analysis of the different morphs, reduction of plates appeared of different magnitude, depending on the site of the plate loss. Reduction of one plate in the middle part of the fish may provide less coverage than reduction in one plate in the keel. However, the prominent bridging of the plates in the keel will most likely account for additional rigidity. Thus, the loss of one plate in the keel should affect the neighbouring lateral plates more than the loss of a lateral plate in front or in the middle of the fish, except those that are overlapping the basal plates and the ascending process of the pelvic girdle. In addition, the position where plate reduction occurs will evidently affect the flexibility of the fish differently. In terms of protection against predators, some lateral plates are probably more important considering coverage of vital organs. The location on the body of the predation attack as well as the dimension of the attack (size and strength of the bite) may vary. Nevertheless, since my intention was to analyse the differences in bone investment between three-spine stickleback in different salinity regimes, effects such as predation type and swimming abilities of sticklebacks will not be further discussed. From the visual analysis of the 3D models, it appeared that the differences in plate reduction should not only be measured in number, but also include the position of the lateral plates when comparing plate reduction in three-spine stickleback.

The comparison of the complete plate morph in different salinity revealed great differences in shape, size and overlapping of plates. The Barents Sea individuals having much larger, bordering and overlapping plates than the individuals from the brackish water and freshwaters populations, this is consistent with the statistical findings, supporting the hypothesis of an alternate armour reduction be decrease in size of lateral plates.

Micro-computed tomography as a method of comparing bone structures in three-spine stickleback

By using Micro-computed tomography bone structures were not only abled to be visually analysed, but also analysed comparing bone density and size parameters of isolated bone structures (Bouxsein *et al.* 2010). This provides information of microstructures and quantify traits in the three-spine stickleback, which further gives an understanding of development and function bone structures. An advantage with µ-CT is its non-destructive features, even allowing repeated scan of living animals. The time of scanning, amount of data and level of analysis depends on the detail of scanning. Higher resolution provides scans that are more detailed; however, this comes as a cost of increased time of the scanning procedure and output data.

Being an expensive method, µ-CT scanning should be optimised between cost and resolution. For smaller animals such as three-spine stickleback, the resolution must be high enough to access differences in the smaller and finer bone structures (Metscher 2009; Bouxsein *et al.* 2010).

Conclusion

Although my results suggest that alternative armour reduction (by decrease in size of plates) is a possibly a plastic response, my results are limited by the number of populations in this study. To strengthen the alternative armour reduction hypothesis, more populations should be compared. In order to understand the genetic, plasticity and selection of alternative armour reduction, populations should be sequenced, lateral plates should be analysed by qualitative measurements, such as µ-CT scan and compared with water samples. In addition, common garden experiments should be conducted.

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Appendix: µCT scanning and reconstruction settings

Scanner=Skyscan1172

Instrument S/N=063

Hardware version=A

Software=Version 1. 5 (build 11)

Home directory=C:\SkyScan

Source Type=Hamamatsu 100/250

Camera=Hamamatsu 10Mp camera

Camera Pixel Size (um)= 11.44

CameraXYRatio=0.9962

Incl.in lifting (um/mm)=-5.4750

[Acquisition]

Data directory=Z:\micro CT\Ongoing projects\stingsild_wiig\fish5_

Filename Prefix=fish5_

Number of Files= 720

Source Voltage (kV)= 59

Source Current (uA)= 167

Number of Rows= 1048

Number of Columns= 2000

Number of connected scans= 4

Number of lines to be reconstructed= 700

Image Pixel Size (um)= 14.87

Object to Source (mm)=181.220

Camera to Source (mm)=278.814

Vertical Object Position (mm)=4.843

Optical Axis (line)= 540

Filter=No filter

Image Format=TIFF

Depth (bits)=16

Screen LUT=0

Exposure (ms)= 295

Rotation Step (deg)=0.500

Frame Averaging=ON (3)

Random Movement=OFF (10)

Use 360 Rotation=YES

Geometrical Correction=ON

Camera Offset=OFF

Median Filtering=ON

Flat Field Correction=ON

Rotation Direction=CC

Scanning Trajectory=ROUND

Type Of Motion=STEP AND SHOOT

Study Date and Time=Jul 23, 2012 14:05:04

Scan duration=00:31:52

[Pre-processing]

Last used post-alignment value [0]=3.000000

Last used post-alignment value [1]=3.000000

Last used post-alignment value [2]=3.500000

Last used post-alignment value [3]=3.500000

[Reconstruction]

Reconstruction Program=NRecon

Program Version=Version: 1.6.4.8

Program Home Directory=C:\Program Files\skysoft

Reconstruction engine=NReconServer

Engine version=Version: 1.6.4

Reconstruction from batch=No

Reconstruction servers= NODE4 NODE1 NODE3 NODE2 FORSKNINGSLAB

Option for additional F4F float format=OFF

Reconstruction mode=Standard

Dataset Origin=Skyscan1172

Dataset Prefix=fish5_~00

Dataset Directory=M:\micro CT\Ongoing projects\stingsild_wiig\fish5_

Output Directory=M:\micro CT\Ongoing projects\stingsild_wiig\fish5_

Time and Date=Jul 23, 2012 17:43:03

First Section=44

Last Section=3105

Reconstruction duration per slice (seconds)=1.521992

Total reconstruction time (3092 slices) in seconds=4706.000000

Postalignment=3.00

Connected Reconstruction (parts)=4

Sub-scan post alignment [0]=3.000000

Sub-scan post alignment [1]=3.000000

Sub-scan post alignment [2]=3.500000

Sub-scan post alignment [3]=3.500000

Section to Section Step=1

Sections Count=3062

Result File Type=BMP

Result File Header Length (bytes)=1134

Result Image Width (pixels)=2000

Result Image Height (pixels)=2000

Pixel Size (um)=14.87122

Reconstruction Angular Range (deg)=360.00

Use 180+=OFF

Angular Step (deg)=0.5000

Smoothing=3

Smoothing kernel=0 (Asymmetrical boxcar)

Ring Artifact Correction=10

Draw Scales=ON

Object Bigger than FOV=OFF

Reconstruction from ROI=OFF

Filter cutoff relative to Nyquisit frequency=100

Filter type=0

Filter type meaning(1)=0: Hamming (Ramp in case of optical scanner); 1: Hann; 2: Ramp; 3: Almost Ramp;

Filter type meaning(2)=11: Cosine; 12: Shepp-Logan; [100,200]: Generalized Hamming, alpha=(iFilter-100)/100

Undersampling factor=1

Threshold for defect pixel mask (%)=0

Beam Hardening Correction (%)=15

CS Static Rotation (deg)=0.0

Minimum for CS to Image Conversion=0.0069

Maximum for CS to Image Conversion=0.3208

HU Calibration=OFF

BMP LUT=0

Cone-beam Angle Horiz.(deg)=9.382551

Cone-beam Angle Vert.(deg)=4.924439

Oversize reference slice=190

Oversize matching slice[0]=887

Oversize matching slice[1]=889

Oversize matching slice[2]=892

Automatic matching=1

Automatic fusion=1

[File name convention]

Filename Index Length=4

Filename Prefix=fish5__rec